DNA-Mediated Gene Transfer to Baby Hamster Kidney Cells

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ABSTRACT. To establish the conditions for the efficient transfer of foreign genes to baby hamster kidney (BHK) cell lines, we investigated the factors that affect the efficiency of transformation of thymidine kinase deficient (*tk*⁻) BHK cells to the *tk*⁺ phenotype. Plasmid pAGO, which carries the herpes simplex virus (HSV) *tk* gene, was used as the donor DNA, and the frequencies of transformation of BHK and mouse L cells under various conditions were compared.

Although the optimal times of exposure to DNA were almost the same, the two kinds of cells had different cell density dependencies; transformation of BHK cells occurred only when a limited number of cells were seeded prior to DNA transfer, whereas there was a high frequency of transformation with mouse L cells over a broader range of cell density. The frequency of transformation increased as the amounts of pAGO DNA increased, and a clear dose dependence was found. The efficiency of the transformation for BHK cells was about 80 *tk*⁺ transformants per 4×10^5 cells per 400 ng of pAGO DNA; about 50 times lower than the efficiency of transformation for mouse L *tk*⁻ cells.

DNA-mediated gene transfer is an important technique for use in investigations of the gene functions of mammalian cells. Specific genes can be introduced stably into cultured cells by calcium phosphate precipitation (transformation) (2-5, 7, 8, 15, 17, 19-23). In addition, the application of this procedure to mutants that have selectable markers would allow us to clone genes of biological interest even when the products of these genes have not been identified (19). Most transformation experiments, however, have been performed with certain mouse cell lines; therefore, applications of this technique to other types of mammalian cells are needed.

A number of *ts* mutants have been isolated from Chinese hamster (CHO) and Syrian hamster (BHK) cell lines (1, 18). Recently, Nishimoto *et al.* (14) established 19 complementation groups of *ts* mutants from BHK21/13 cells for cell reproduction. In hopes of using these mutants to clone genes for cell growth, we have studied the conditions needed for the efficient transfer of genes to BHK cell lines.

Abbreviations used: BHK, baby hamster kidney; BrdU, 5-bromodeoxyuridine; HSV, herpes simplex virus; kb, kilobase pairs; MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; *tk*, thymidine kinase; *ts*, temperature-sensitive; *tk*⁺, cell line that has *tk* enzyme and *tk*⁻, cell line that does not have *tk* enzyme.

The herpes simplex virus *tk* gene was used to transform *tk*-deficient BHK cells. Various factors that affect gene transfer also were investigated. Throughout the experiments, comparisons were made between BHK and mouse L cells, the latter known to be proficient recipients.

MATERIALS AND METHODS

Cell lines. The Syrian hamster cell line, B1, a *tk*-defective derivative of BHK21/13, (9) was provided by Dr. Claudio Bacilico. BN2-BU2 is a *tk*-deficient, *ts* growth mutant of BHK that was isolated from strain BN2 (13) as follows: BN2 cells were seeded at 2×10^5 cells/50-mm dish, after which they were incubated at 33.5°C for 2 days then exposed to 1 µg/ml MNNG for 16 h. Following the removal of MNNG, the cells were incubated in fresh medium to confluence, after which they were trypsinized, then replated and incubated at 33.5°C in the presence of 50 µg/ml BrdU. Four weeks later, one of the colonies that had formed was designated BN2-BU2 and used for the experiments reported here. These BN2-BU2 cells could not grow in HAT medium and were resistant to 100 µg/ml of BrdU. A *tk*-deficient mouse strain, *Ltk*⁻ clone 1D, (6) also was used.

Cell culture. Cells deficient in *tk* were grown in an incubator in Dulbecco's modified Eagle's medium supplemented with 10% calf serum and 10 µg/ml of BrdU in a humidified atmosphere containing 10% CO₂ at 37.5°C (for *Ltk*⁻ and B1) or at 33.5°C (for BN2-BU2). HAT medium containing 10⁻⁴ M hypoxanthine, 10⁻⁵ M aminopterin and 10⁻⁵ M thymidine was used for the selection of *tk*⁺ transformants.

DNA source. High molecular weight carrier-DNA was isolated from B1 cells according to the procedure of the Cold Spring Harbor Laboratory (M. Wigler, personal communication). Briefly, confluent, healthy B1 cells were harvested, then resuspended in 10 mM Tris-10 mM EDTA-100 µg/ml proteinase K (pH 7.5) at 1×10^7 cells/ml and lysed by the addition of SDS to 0.5%. The lysate was incubated at 37°C for 6–12 h then treated sequentially with phenol and chloroform.

High molecular weight DNA was isolated by mixing the aqueous phase with 2 volumes of cold ethanol then immediately removing the DNA precipitate that formed. The precipitate was washed with 70% ethanol then dissolved in 10 mM Tris-10 mM EDTA (pH 7.5). The DNA solution was treated with heated RNase A (Sigma, Co. Ltd.) at 20 µg/ml and RNase T1 (Sigma, Co. Ltd.) at 1 unit/ml for 4 h at 37°C. The mixture was brought to 0.2 M NaCl and 0.1% SDS after which it was treated with 100 µg/ml of proteinase K for 2 h at 37°C. DNA was extracted with phenol and chloroform then isolated by ethanol precipitation. The DNA clot was dissolved in sterile, 1 mM Tris-1 mM EDTA (pH 7.5) at a final concentration of 1 mg of DNA per milliliter.

Plasmid pAGO, donated by Dr. F. Corbere-Garapin, was used as the donor HSV *tk* gene (2). A 200-ml culture of *E. coli* HB101 carrying pAGO was grown in broth. Plasmids were amplified by shaking the culture with 150 µg/ml of added chloramphenicol for 15 h. The cells were pelleted then resuspended in 7 ml of lysis solution (5 mg/ml lysozyme in 0.25 M Tris-HCl, pH 8.5). This cell suspension was kept at 0°C for 5 min, after which 0.2 ml pancreatic RNase (10 mg/ml in 50 mM sodium acetate, pH 4.8) and 1.7 ml Triton mixture (0.3% Triton X100, 0.15 M Tris-HCl, 0.18 M EDTA, pH 8.0) were added to it. The suspension then was swirled gently and incubated at 0°C for 15 min, after which it was spun at 36,000 rpm for 30 min in a RP65 rotor in Hitachi centrifuge. Its supernatant solution was shaken with phenol, then the DNA was precipitated with ethanol. This DNA was purified by banding it on an ethidium bromide-CsCl density gradient then dissolving the band in 10 mM

Tris-1 mM EDTA (pH 7.9).

DNA-mediated gene transfer. The DNA-calcium phosphate precipitate was prepared immediately before use (5, 20). High molecular weight carrier-DNA (20 μ g) and various amounts of purified plasmid DNA were mixed well in 2.1 ml of sterile water. Then, 0.3 ml of 2 M CaCl_2 was added and mixed well (final volume, 2.5 ml). The DNA calcium solution was added by drops to 2.5 ml of twice-concentrated HEPES buffer solution (280 mM NaCl, 50 mM HEPES, Sigma, Co. Ltd, and 1.5 mM sodium phosphate). The pH was adjusted to 7.10 and air was bubbled through the flask. This mixture was allowed to stand at room temperature for 30 min before overlaying it onto the cells.

Cells were seeded at various concentrations (usually 5×10^5 cells) in a 100-mm dish and grown for 24 h in 10 ml of growth medium. One milliliter of the suspension of the DNA-calcium phosphate precipitate was added by drops over the surface of the medium that covered the cells. After various adsorption periods (12 to 24 h) the medium was removed by aspiration, then the cells were fed 10 ml of fresh growth medium. After another 24 to 32 h of culture, the medium was removed as before and the cells fed HAT selection medium. Cultures thereafter were fed HAT medium every 3 days for 2 to 3 weeks. Colonies were scored after they had been fixed with 10% formaldehyde in saline and had been stained with 0.3% crystalviolet.

RESULTS

The efficiencies of DNA-mediated gene transfer to mouse L and BHK cells were compared under a variety of conditions. Of the many factors which might influence this transfer, we studied the effects of DNA-adsorption time, the density of recipient

TABLE 1. EFFECT OF DNA ADSORPTION TIME ON TRANSFORMATION EFFICIENCIES

Expt. No.	Recipient cells	Time of exposure (hours)	No. of tk^+ cells per dish
I	Mouse Ltk^-	8	120
		12	210
		16	240
		20	205
		24	210
II	BHK B1	4	48
		8	80
		12	95
		16	60
		20	65

In experiment I, Ltk^- cells were seeded at 5×10^5 cells per 100-mm dish. After incubation for 24 h at 37.5°C, cultures were fed fresh medium and incubated for an additional 4 h. One milliliter of DNA-calcium phosphate precipitate that contained 20 ng of pAGO DNA and 20 μ g of high molecular weight carrier-DNA was added by drops to the medium covering the cultures of Ltk^- cells. This mixture was allowed to stand for the periods indicated. After the DNA had been adsorbed, the medium was changed to fresh growth medium. The cultures then were incubated for 24 h at 37.5°C, after which they were fed HAT medium. About 2 weeks later, the tk^+ colonies that had formed were fixed and stained. The values shown are the average number of tk^+ colonies for three independent experiments. In Experiment II, B1 cells were seeded at 4×10^5 cells per 100-mm dish, then 400 ng of pAGO DNA and 20 μ g of high molecular weight carrier-DNA were added. Other procedures and conditions were the same as in Experiment I.

cells and the dose of the added DNA.

Adsorption time. *Ltk*⁻ cells were seeded at a density of 5×10^5 cells/100-mm dish and B1 cells at 4×10^5 cells/100-mm dish. About 4 to 8 h prior to DNA transfer, cultures were fed fresh growth medium. PAGO DNA was added at 20 ng/dish to cultures of *Ltk*⁻ cells and at 400 ng/dish to cultures of B1 cells, after which the cells were exposed to DNA for various periods. Although the efficiency of transformation of B1 cells was very low in comparison to that of *Ltk*⁻ cells, the optimal times for DNA adsorption did not differ much (Table 1); the largest number of transformants was found at 16 hours for *Ltk*⁻ cells and at 12 h for B1 cells.

A similar experiment was performed with a *ts* mutant of BHK, BN2-BU2. The number of *tk*⁺ transformants increased as the DNA adsorption time increased and the greatest transformation efficiency was reached when the cells were exposed to DNA for 28 h (Fig. 1). The longer adsorption time needed by BN2-BU2 cells may be related to the longer generation time for these cells (30 h) as compared to the generation time for B1 cells (17 h).

Cell density. Recipient *tk*⁻ cells were seeded at various concentrations (0.5 to 8×10^5 cells/100-mm dish) then incubated for 24 h. B1 cells were sparse in cultures in which less than 2.5×10^5 cells had been seeded, but cultures in which more than 5×10^5 cells had been seeded became confluent. In contrast, cultures of *Ltk*⁻ cells were sparse even at 5×10^5 cells/100 mm dish. A fixed amount of pAGO DNA was applied to cultures with different cell densities and the number of *tk*⁺ transformants that appeared was scored.

Fig. 1. Effect of adsorption times on transformation efficiency in BN2-BU2 cells. Logarithmically growing cells were treated with trypsin and seeded at 5×10^5 cells in a 100-mm dish containing 10 ml of growth medium. After incubation at 33.5°C for 24 h, the medium was changed to fresh growth medium. After another 8 h of culture, 1 ml of the DNA-calcium phosphate precipitate containing 500 ng of pAGO and 20 µg of carrier DNA was added by drops, and the medium was allowed to stand for various periods. After DNA adsorption, this medium was exchanged for fresh growth medium. The cells then were incubated at 33.5°C for an additional 32 h after which the medium was changed to HAT medium. After 4 weeks, the number of *tk*⁺ colonies were scored. Each point is the average value for *tk*⁺ transformant colonies from two dishes.

Fig. 2. Effect of cell density on transformation efficiency. Cells were seeded at the concentrations indicated on the abscissa. The other experimental conditions used are described in Fig. 1 and Table 1, but the period for DNA adsorption was fixed at 16 h for *Ltk*⁻, 12 h for BI and 24 h for BN2-BU2 cells. The relative numbers of *tk*⁺ transformants per dish are shown. (a) mouse *Ltk*⁻ (highest value: 146 transformants per dish). (b) BHK cells. $\circ$ — $\circ$, BI (highest value: 78 transformants per dish); $\bullet$ — $\bullet$, BN2-BU2 (47 transformants per dish).

Different patterns of dependency on cell density were found for the mouse and hamster cell lines (Fig. 2). With *Ltk*⁻ cells, the number of transformants increased as the number of recipient cells increased, but the curve leveled off when the initial number of cells exceeded 2.5×10^5 cells/dish. BHK cells, however, had a narrow range of optimal cell density; the highest efficiencies of transformation were reached at an initial cell density of 2.5×10^5 cells/dish for BI cells and at 5×10^5 cells/dish for BN2-BU2 cells. No transformant appeared when more than 8×10^5 BHK cells were seeded in a dish.

To see whether cell density affects the colony formation of *tk*⁺ transformants, we seeded BI cells at two cell-densities, after which the cultures were incubated for 25 h then exposed to pAGO DNA. After DNA had been adsorbed, the cells were collected and replated at different cell densities. The results given in Table 2 show that many transformants were formed when cultures whose initial cell density had been 4×10^5 cells/dish were replated at 2×10^6 cell/dish after DNA adsorption. But, when cells had been seeded at 8×10^5 cells/dish, only few transformants were formed even though replating was done at 5×10^5 cells/dish after DNA adsorption. Thus, cell density appears to have been critical during the period when cells took up the DNA-calcium precipitate.

DNA dose-dependence of transformation. Using the conditions established above we studied the influence of the dosage of pAGO DNA on transformation frequency.

TABLE 2. EFFECT ON TRANSFORMATION EFFICIENCY OF REPLATING BI CELLS AFTER DNA ADSORPTION

No.	Number of cells seeded prior to DNA transfer	Amount of pAGO DNA	Number of cells prior to HAT selection	Average Number* of <i>tk</i> ⁺ colonies
	cells/dish	ng/dish	cells/dish	colonies/dish
I	4×10^5	0	1×10^6	0
II	4×10^5	400	1×10^6	41
III	4×10^5	400	2×10^6	53
IV	8×10^5	400	1.5×10^6	1
V	8×10^5	400	5×10^5	2

Nos. I and II: Cells were seeded at 4×10^5 cells/100-mm dish. Cultures were not replated after DNA adsorption. No. III: Cells were seeded in 4 dishes at 4×10^5 cells/100-mm dish. After DNA had been adsorbed, cultures were incubated for 24 h in fresh medium. Cells then were collected and replated at 2×10^6 cells/dish; that is, cells from two dishes were seeded in one dish. No. IV: Cells were seeded at 8×10^5 cells/100-mm dish. Cells were not replated after DNA adsorption, and the colonies formed were tiny. No. V: Cells were seeded at 8×10^5 cells/dish. After DNA had been adsorbed, cultures were incubated for 24 h in fresh medium, then the cells were replated at 5×10^5 cells/dish. *The total number of *tk*⁺ colonies divided by the number of dishes.

Fig. 3. Dose-response curves for transformation. (a) *Ltk*⁻ cells were seeded at 5×10^5 cells in a 100-mm dish then treated as described in Table 1. DNA-calcium phosphate precipitate containing various amounts of pAGO DNA and 20 μ g of carrier DNA was added, and the cells were allowed to adsorb DNA for 16 h. (b) BI cells were seeded at 4×10^5 cells per dish and BN2-BU2 cells at 5×10^5 cells per dish. These cells were exposed to DNA precipitate that contained various amounts of pAGO DNA and 20 μ g of carrier DNA for 12 h (BI cells) (○—○) or 24 h (BN2-BU2) (●—●). The other procedures and conditions used are described in Table 1 and Fig. 1.

Results with mouse *Ltk*⁻ cells are shown in Fig. 3a. The increase in the number of *tk*⁺ colonies was approximately proportional to the pAGO DNA added up to about 20 ng per 100-mm dish. An efficiency of transformation of about 10 colonies per nanogram of pAGO DNA was calculated from this curve.

Similar dose-response curves were obtained with the two BHK cell lines, but large amounts of pAGO DNA were required to obtain similar values. B1 cells produced 71 *tk*⁺ transformants when treated with 400 ng of pAGO DNA per dish, and BN2-BU2 cells produced 35 *tk*⁺ transformants. Thus, the transformation efficiencies of these cell lines were 100 to 180 *tk*⁺ colonies per microgram of pAGO DNA, approximately 50~100-fold lower than the value for mouse *Ltk*⁻ cells.

DISCUSSION

We have used the calcium-phosphate precipitation method to study the factors that govern the transfer of the *tk* gene to mouse *Ltk*⁻ cells. The results obtained were in general agreement with those of previous studies (5, 8), and the transformation frequencies found in these experiments were almost the same as the values obtained previously for *Ltk*⁻ cells (2, 4, 17, 23). We now have established the procedures and techniques for genetic transformation and have extended our studies to BHK cells, from which cells many *ts* growth mutants have been isolated (1, 12, 14). We investigated the effects of various factors on the transformation of BHK cells and compared them with the results obtained with *Ltk*⁻ cells.

The optimal adsorption time for gene transfer did not differ much between *Ltk*⁻ and B1 cells, but B1 cells were more sensitive to high calcium concentrations than were *Ltk*⁻ cells. B1 cells tended to die and come off the plates after prolonged exposure to calcium phosphate.

Cell density was critical for the transformation of B1 cells, but not for that of *Ltk*⁻ cells. A narrow range of optimal cell density was found for BN2-BU2 cells, a *tk*-deficient derivative of the *ts* BHK cell line. BHK cells are fibroblastic and flat; thus, they may be more sensitive to contact inhibition than *Ltk*⁻ cells. This difference in morphology may be related to the difference in the dependency on cell density for L and BHK cells. We are interested in discovering whether CHO and human cells have similar dependencies on cell density for gene transfer because these cell lines have a morphology similar to that of BHK21 and the efficiency of genetic transformation was low in these lines (4, 8, 15).

Cell density does not seem to affect the selectivity of *tk*⁺ transformants because the number of *tk*⁺ transformants did not change when recipient cells were replated after DNA adsorption (Table 2).

Which step of transformation is critically affected by cell density? For transformation by gene transfer, the DNA added must pass through several barriers; the outer cell-membrane, and the lysosome and nuclear membranes (16). Furthermore, DNA must be integrated into the host's chromosomal DNA in a way appropriate for expressing the *tk* gene (7). These steps must be completed during the adsorption and expression time following the addition of DNA. Thus, the effect of cell density probably is due to cell to cell contact.

Loyter *et al.* (10) recently demonstrated that HeLa cells take up the DNA-calcium phosphate precipitate very quickly although their transformation frequency is low. They found the added DNA in the cytoplasm of most recipient cells, but very little

was present in the nuclei, and they suggested that the transfer of the added DNA from the cytoplasm to the nuclei is a critical step in transformation. If so, in BHK cells this step(s) may be affected by cell density. This possibility can not be excluded because we have not yet examined whether the added DNA is present in the cytoplasm of recipient cells.

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